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Vaccine antigens of Moraxella

FIELD OF THE INVENTION

The present invention relates to antigens of *Moraxella*, in particular, *Moraxella bovis*, nucleic acid sequences encoding these antigens and formulations for use in raising an immune response against *Moraxella*.

BACKGROUND OF THE INVENTION

Infectious bovine keratoconjunctivitis (IBK) is an economically important disease of cattle caused by the Gram-negative coccobacillus *Moraxella bovis*. More commonly known as pinkeye, IBK is a highly contagious ocular infection which may range from mild conjunctivitis to severe ulceration, corneal perforation and blindness. Therapeutic and preventative measures have limited success in controlling IBK and a vaccine which will prevent the disease is required. A number of factors contribute to the virulence of the organism, the two most important attributes so far identified are the expression of pili, and the ability to produce haemolysin. Seven different serogroups of *M. bovis* strains isolated in Australia, Great Britain and the USA have been characterised, based on pilus types (1). An efficacious pilus-based vaccine must contain a sufficient quantity of pili from all seven serotypes to be fully protective, because of a lack of cross protection between serotypes (2, 3). Expression of all seven pilus serotypes at levels high enough to be useful for commercial vaccine preparation has not been achieved.

The ideal vaccine candidate to stimulate protection against *M. bovis* would be a molecule that is highly-conserved among all strains of this species. Possible candidates are haemolysin, protease, lipase and/or phospholipase (4) enzymes produced by *M. bovis*. For example, a partially purified cell-free supernatant from one haemolytic strain of *M. bovis* has been shown to confer significant protection against heterologous, wild-type challenge (5). The possibility that a haemolysin could be conserved across all seven serotypes of *M. bovis* makes it a potential vaccine candidate against IBK. However, researchers have so far been unable to either clone the gene encoding the haemolysin or purify the protein to homogeneity. Nevertheless, any or all of these molecules, alone or in combination, could prove useful for the generation of an effective vaccine against IBK.

SUMMARY OF THE INVENTION

In a first aspect the present invention consists in a polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO. 1 from amino acid 37 to 1114, or a sequence having at least 50% identity thereto, or a functional fragment thereof.

In a preferred embodiment the polypeptide has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 1.

In a further preferred embodiment of the first aspect of the present invention the polypeptide has protease activity.

In a second aspect the present invention consists in a nucleic acid molecule, the nucleic acid molecule encoding the polypeptide of the first aspect of the present invention.

In a third aspect the present invention consists in a nucleic acid molecule comprising a sequence as set out in SEQ. ID. NO. 2 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.

In a preferred embodiment the nucleic acid molecule has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 2.

In a fourth aspect the present invention consists in a composition for use in raising an immune response in an animal, the composition comprising the polypeptide of the first aspect of the present invention or the nucleic acid sequence of the second aspect of the present invention and optionally a carrier and/or adjuvant.

In a fifth aspect the present invention consists in a polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO. 3 from amino acid 26 to 616, or a sequence having at least 50% identity thereto, or a functional fragment thereof.

In a preferred embodiment the polypeptide has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 3 from amino acid 26 to 616.

In a further preferred embodiment of the fifth aspect the polypeptide has lipase activity.

In a sixth aspect the present invention consists in a nucleic acid molecule, the nucleic acid molecule encoding the polypeptide of the fifth aspect of the present invention.

In a seventh aspect the present invention consists in a nucleic acid molecule comprising a sequence as set out in SEQ. ID. NO. 4 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.

In a preferred embodiment the nucleic acid molecule has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 4.

In an eighth aspect the present invention consists in a composition for use in raising an immune response in an animal, the composition comprising the polypeptide of the fifth aspect of the present invention or the nucleic acid sequence of the sixth aspect of the present invention and optionally a carrier and/or adjuvant.

In a ninth aspect the present invention consists in a polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO. 5, or a sequence having at least 60% identity thereto, or a functional fragment thereof.

In a preferred embodiment the polypeptide has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 5.

In a further preferred embodiment of the ninth aspect the polypeptide has haemolysin activity.

In a tenth aspect the present invention consists in a nucleic acid molecule, the nucleic acid molecule encoding the polypeptide of the ninth aspect of the present invention.

In an eleventh aspect the present invention consists in a nucleic acid molecule comprising a sequence as set out in SEQ. ID. NO. 6 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.

In a preferred embodiment the nucleic acid molecule has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 6.

In a twelfth aspect the present invention consists in a composition for use in raising an immune response in an animal, the composition comprising

the polypeptide of the ninth aspect of the present invention or the nucleic acid sequence of the tenth aspect of the present invention and optionally a carrier and/or adjuvant.

The term "functional fragment" as used herein is intended to cover fragments of the polypeptide which retain at least 10% of the biological activity of the complete polypeptide. In particular this term is used to encompass fragments which show immunological cross-reactivity with the entire polypeptide, eg ligands which react with the fragment also react with the complete polypeptide.

In a thirteenth aspect the present invention consists in a composition for use in raising an immune response in an animal directed against *Moraxella*, the composition comprising at least one polypeptide selected from the group consisting of the polypeptides of the first, fifth and ninth aspects of the present invention and optionally including an adjuvant or carrier.

In a preferred embodiment the composition includes the polypeptide of the ninth aspect of the present invention and either one of, or preferably both, the polypeptides of the first and fifth aspects of the present invention.

In a preferred embodiment the *Moraxella* is *M. bovis or M. catarrhalis*, most preferably *M. bovis*.

In a fourteenth aspect the present invention consists in an antibody raised against a polypeptide selected from the group consisting of the polypeptides of the first, fifth and ninth aspects.

As will be readily appreciated by the person skilled in this field the polypeptides and antibodies of the present invention and probes derived from the nucleotide sequences can be used as diagnostic reagents in determining *Moraxella*, in particular, *M. bovis* infection. For example, the polypeptides and antibodies can be used in ELISA based assays whilst the probes can be used in PCR based assays. The probes will be of a length to provide the required level of specificity and will typically be at least 18 nucleotides in length.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Nucleotide and amino acid sequence of a protease from *M. bovis* Dalton 2d. A putative promoter sequence is singly underlined. A putative ribosome binding site is shown in bold and underlined. A putative start codon is shown in bold. Putative transcription terminator sequences are indicated by inverted arrows. Numbering for both the nucleotide and amino acid sequences are shown on the left hand side.

Figure 2: Nucleotide and amino acid sequence of a lipase from *M. bovis* Dalton 2d. A putative promoter sequence is singly underlined. A putative ribosome binding site is shown in bold and underlined. A putative start codon is shown in bold. Putative transcription terminator sequences are indicated by inverted arrows. Numbering for both the nucleotide and amino acid sequences are shown on the left hand side.

Figure 3: Heat stability of the lipase from *M.bovis* when expressed in its recombinant form (pMB1/MC1061). (Heating carried out at 90°C).

Figure 4: Comparison of growth rate and expression levels of the lipase of *M.bovis* when in its (i) native form and (ii) recombinant form. The growth rate is shown as solid bars and the lipase expression levels as open diamonds.

Figure 5: Nucleotide and amino acid sequence of the A subunit of the RTX toxin from *M. bovis* Dalton 2d. A putative ribosome binding site is shown in bold and underlined. A putative start codon is shown in bold. Upstream of the A subunit open reading frame is a portion of the coding region for the C subunit (nucleotide 1 to 195) (corresponding amino acid sequence shown in SEQ ID NO:8) and downstream of the A subunit is a small portion of the B subunit coding region (nucleotide 3080 to 3250) (corresponding amino acid sequence shown in SEQ ID NO:9) . Numbering for both the nucleotide and amino acid sequences are shown on the left hand side.

DETAILED DESCRIPTION OF THE INVENTION

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following non-limiting Examples.

General Molecular Biology

Unless otherwise indicated, the recombinant DNA techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (Editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present) and are incorporated herein by reference.

Protein Variants

Amino acid sequence variants can be prepared by introducing appropriate nucleotide changes into DNA, or by *in vitro* synthesis of the desired polypeptide. Such variants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final protein product possesses the desired characteristics. The amino acid changes also may alter post-translational processes such as altering the membrane anchoring characteristics, altering the intra-cellular location by inserting, deleting or otherwise affecting the transmembrane sequences of the native protein, or modifying its susceptibility to proteolytic cleavage.

In designing amino acid sequence variants, the location of the mutation site and the nature of the mutation will depend on characteristic(s) to be modified. The sites for mutation can be modified individually or in

series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of other ligands adjacent to the located site.

A useful method for identification of residues or regions for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (*Science* (1989) **244**: 1081-1085*)*. Here, a residue or group of target residues are identified (e.g., charged residues such as Arg. Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimise the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants; the location of the mutation site and the nature of the mutation. These may represent naturally occurring alleles or predetermined mutant forms made by mutating the DNA either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon the characteristic to be modified.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues and typically about 1 to 5 contiguous residues.

Amino acid sequence insertions include amino and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Other insertional variants include the fusion of the N- or C-terminus of the proteins to an immunogenic polypeptide e.g. bacterial polypeptides such as betalactamase or an enzyme encoded by the *E. coli trp* locus, or yeast protein, bovine serum albumin, and chemotactic polypeptides. C-terminal fusions with proteins having a

long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, are included.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the protein molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s). Other sites of interest are those in which particular residues obtained from various species are identical. These positions may be important for biological activity. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

TABLE 1

Original Residue	**Exemplary Substitutions**	**Preferred Substitutions**
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe norleucine	leu
Leu (L)	norleucine, ile; val; met; ala; phe	ile

Original Residue	Exemplary Substitutions	Preferred Substitutions
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile;	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr	thr
Thr (T	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Mutants, Variants and Homology - Proteins

Mutant polypeptides will possess one or more mutations which are deletions, insertions, or substitutions of amino acid residues. Mutants can be either naturally occurring (that is to say, purified or isolated from a natural source) or synthetic (for example, by performing site-directed mutagensis on the encoding DNA). It is thus apparent that polypeptides of the invention can be either naturally occurring or recombinant (that is to say prepared using recombinant DNA techniques).

An allelic variant will be a variant that is naturally occurring within an individual organism.

Protein sequences are homologous if they are related by divergence from a common ancestor. Consequently, a species homologue of the protein will be the equivalent protein which occurs naturally in another species. Within any one species a homologue may exist as numerous allelic variants, and these will be considered homologues of the protein. Allelic variants and species homologues can be obtained by following standard techniques known to those skilled in the art. Preferred species homologues include those obtained from representatives of the same Phylum, more preferably the same Class and even more preferably the same Order.

A protein at least 50% identical, as determined by methods well known to those skilled in the art (for example, the method described by Smith, T.F. and Waterman, M.S. (1981) Ad. Appl Math., 2: 482-489, or Needleman, S.B. and Wunsch, C.D. (1970) J. Mol. Biol., 48: 443-453), to that of the present invention are included in the invention, as are proteins at least 70% or 80% and more preferably at least 90% identical to the protein of the present invention. This will generally be over a region of at least 20, preferably at least 30, contiguous amino acids.

Mutants, Variants and Homology - Nucleic Acids

Mutant polynucleotides will possess one or more mutations which are deletions, insertions, or substitutions of nucleotide residues. Mutants can be either naturally occurring (that is to say, isolated from a natural source) or synthetic (for example, by performing site-directed mutagensis on the DNA). It is thus apparent that polynucleotides of the invention can be either naturally occurring or recombinant (that is to say prepared using recombinant DNA techniques).

An allelic variant will be a variant that is naturally occurring within an individual organism.

Nucleotide sequences are homologous if they are related by divergence from a common ancestor. Consequently, a species homologue of the polynucleotide will be the equivalent polynucleotide which occurs naturally in another species. Within any one species a homologue may exist as numerous allelic variants, and these will be considered homologues of the polynucleotide. Allelic variants and species homologues can be obtained by following standard techniques known to those skilled in the art. Preferred species homologues include those obtained from representatives of the same Phylum, more preferably the same Class and even more preferably the same Order.

A polynucleotide at least 70% identical, as determined by methods well known to those skilled in the art (for example, the method described by Smith, T.F. and Waterman, M.S. (1981) Ad. Appl Math., 2: 482-489, or Needleman, S.B. and Wunsch, C.D. (1970) J. Mol. Biol., 48: 443-453), to that of the present invention are included in the invention, as are proteins at least 80% or 90% and more preferably at least 95% identical to the

polynucleotide of the present invention. This will generally be over a region of at least 60, preferably at least 90, contiguous nucleotide residues.

Antibody Production

The term "antibody" should be construed as covering any specific binding substance having a binding domain with the required specificity. Thus, the term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide including an immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules including an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included.

Antibodies, either polyclonal or monoclonal, which are specific for a protein of the present invention can be produced by a person skilled in the art using standard techniques such as, but not limited to, those described by Harlow et al. Antibodies: A Laboratory Manual, Cold Springs Harbor Laboratory Press (1988), and D. Catty (editor), Antibodies: A Practical Approach, IRL Press (1988).

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of a protein. For the production of polyclonal antibodies, a number of host animals are acceptable for the generation of antibodies by immunization with one or more injections of a polypeptide preparation, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response in the host animal, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminium hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, oil emulsions, keyhole lympet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

A monoclonal antibody to an epitope of a protein may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256, 493-497), and the more recent human B-cell hybridoma technique (Kesber *et al.* 1983, Immunology Today 4:72) and EBV-hybridoma technique (Cole *et al.* 1985, Monoclonal Antibodies and

Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" by splicing the genes from an antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity may be used (Morrison *et al.* 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger *et al.* 1984 Nature 312:604-608; Takeda *et al.* 1985 Nature 31:452-454). Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce 4-specific single chain antibodies.

Recombinant human or humanized versions of monoclonal antibodies are a preferred embodiment for human therapeutic applications. Humanized antibodies may be prepared according to procedures in the literature (e.g. Jones *et al.* 1986, Nature 321:522-25; Reichman *et al.* 1988, Nature 332:323-27; Verhoeyen *et al.* 1988, Science 239:1534-36). The recently described "gene conversion mutagenesis" strategy for the production of humanized monoclonal antibody may also be employed in the production of humanized antibodies (Carter *et al.* 1992 Proc. Natl. Acad. Sci. U.S.A. 89:4285-89). Alternatively, techniques for generating the recombinant phage library of random combinations of heavy and light regions may be used to prepare recombinant antibodies (e.g. Huse *et al.* 1989 Science 246:1275-81).

Antibody fragments which contain the idiotype of the molecule such as Fu $F(ab^1)$ and $F(ab^2)$ may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab) E2 fragment which can be produced by pepsin digestion of the intact antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragment, and the two Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Alternatively, Fab expression libraries may be constructed (Huse *et al.* 1989, Science 246:1275-1281) to allow rapid and easy cloning of a monoclonal Fab fragment with the desired specificity to a protein.

Adjuvants and Carriers

Pharmaceutically acceptable carriers or diluents include those used in compositions suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. They are

non-toxic to recipients at the dosages and concentrations employed. Representative examples of pharmaceutically acceptable carriers or diluents include, but are not limited to water, isotonic solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline) and can also contain one or more of, mannitol, lactose, trehalose, dextrose, glycerol, ethanol or polypeptides (such as human serum albumin). The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy.

As mentioned above the composition may include an adjuvant. As will be understood an "adjuvant" means a composition comprised of one or more substances that enhances the immunogenicity and efficacy of a vaccine composition. Non-limiting examples of suitable adjuvants include squalane and squalene (or other oils of animal origin); block copolymers; detergents such as Tween®-80; Quil® A, mineral oils such as Drakeol or Marcol, vegetable oils such as peanut oil; *Corynebacterium*-derived adjuvants such as *Corynebacterium parvum*; *Propionibacterium*-derived adjuvants such as *Propionibacterium acne*; *Mycobacterium bovis* (Bacille Calmette and Guerin or BCG); interleukins such as interleukin 2 and interleukin 12; monokines such as interleukin 1; tumour necrosis factor; interferons such as gamma interferon; combinations such as saponin-aluminium hydroxide or Quil-A aluminium hydroxide; liposomes; ISCOM adjuvant; mycobacterial cell wall extract; synthetic glycopeptides such as murarmyl dipeptides or other derivatives; Avridine; Lipid A derivatives; dextran sulfate; DEAE-Dextran or with aluminium phosphate; carboxypolymethylene such as Carbopol' EMA; acrylic copolymer emulsions such as Neocryl A640 (e.g. U.S. Pat. No. 5,047,238); vaccinia or animal poxvirus proteins; sub-viral particle adjuvants such as cholera toxin, or mixtures thereof.

Gene/DNA Isolation

The DNA encoding a protein may be obtained from any cDNA library prepared from tissue believed to express the gene mRNA and to express it at a detectable level. DNA can also be obtained from a genomic library.

Libraries are screened with probes or analytical tools designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include monoclonal or polyclonal

antibodies that recognize and specifically bind the protein; oligonucleotides of about 20-80 bases in length that encode known or suspected portions of cDNA from the same or different species; and/or complementary or homologous cDNAs or fragments thereof that encode the same or a hybridizing gene. Appropriate probes for screening genomic DNA libraries include, but are not limited to, oligonucleotides; cDNAs or fragments thereof that encode the same or hybridizing DNA including expressed sequence tags and the like; and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook *et al.*

An alternative means to isolate a gene encoding the protein of interest is to use polymerase chain reaction (PCR) methodology as described in section 14 of Sambrook *et al.* This method requires the use of oligonucleotide probes that will hybridize to the gene.

The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The actual nucleotide sequence(s) is usually based on conserved or highly homologous nucleotide sequences or regions of the gene. The oligonucleotides may be degenerate at one or more positions. The use of degenerate oligonucleotides may be of particular importance where a library is screened from a species in which preferential codon usage in that species is known. The oligonucleotide must be labelled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labelling is to use (α-^{32}P)- dATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labelling.

DNA encompassing all the protein coding sequence is obtained by screening selected cDNA or genomic libraries, and if necessary, using conventional primer extension procedures as described in section 7.79 of Sambrook *et al.*, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Another alternative method for obtaining the gene of interest is to chemically synthesize it using one of the methods described in Fingels *et al.* (*Agnew Chem. Int. Ed. Engl. 28:* 716-734, 1989). These methods include

triester, phosphite, phosphoramidite and H-Phosphonate methods, PCR and other autoprimer methods, and oligonucleotide syntheses on solid supports. These methods may be used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available, or alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue.

Substantially Purified

By "substantially purified" we mean a polypeptide that has been separated from lipids, nucleic acids, other polypeptides, and other contaminating molecules.

Hybridisation

The polynucleotide sequence of the present invention may hybridise to the respective sequence set out SEQ. ID. NOS. 2, 4, or 6 under high stringency. As used herein, stringent conditions are those that (i) employ low ionic strength and high temperature for washing after hybridization, for example, 0.1 x SSC and 0.1% (w/v) SDS at 50°C; (ii) employ during hybridization conditions such that the hybridization temperature is 25°C lower than the duplex melting temperature of the hybridizing polynucleotides, for example 1.5 x SSPE, 10% (w/v) polyethylene glycol 6000, 7% (w/v) SDS, 0.25 mg/ml fragmented herring sperm DNA at 65°C; or (iii) for example, 0.5M sodium phosphate, pH 7.2, 5mM EDTA, 7% (w/v) SDS and 0.5% (w/v) BLOTTO at 70°C; or (iv) employ during hybridization a denaturing agent such as formamide, for example, 50% (v/v) formamide with 5 x SSC, 50mM sodium phosphate (pH 6.5) and 5 x Denhardt's solution (32) at 42°C; or (v) employ, for example, 50% (v/v) formamide, 5 x SSC, 50mM sodium phosphate (pH 6.8), 0.1% (w/v) sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50μg/ml) and 10% dextran sulphate at 42°C.

EXAMPLE 1

This example describes the cloning and characterisation of a protease from *Moraxella bovis*.

Bacteria and construction of a genomic library

Moraxella bovis strain Dalton 2d was a field isolate collected from a bovine eye and characterised by CSIRO Animal Health, Parkville, Australia (6). *Escherichia coli* strain DH5α has been previously described (7, 8).

All enzymes were purchased from Promega (Madison, WI, USA) except where otherwise noted.

General cloning and DNA techniques were as described (9) unless otherwise noted.

A genomic library was constructed by carrying out partial *Sau*3A digests on genomic DNA extracted from *M. bovis* strain Dalton 2d using a CTAB method which is outlined below. This DNA was size fractionated using a NaCl gradient (10) and ligated with the cosmid cloning vector pHC79 (11) which had been previously digested with *Bam*HI. This DNA was packaged into lambda bacteriophage heads using the Packagene Lambda DNA packaging system (Promega, Madison,WI, USA) and this was used to transduce the *E. coli* strain DH5α. The library was stored in 96 well trays (50% glycerol / luria broth / ampicillin (50μg/ml)) at -70°C.

CTAB genomic DNA extraction from *M. bovis*

A 5ml brain heart infusion (BHI) (Oxoid Ltd., Basingstoke, Hampshire, U.K.) broth was inoculated with a colony of Dalton 2d taken from a fresh overnight culture on horse blood agar and incubated with shaking at 37°C for 6 hours. This culture was used to inoculate 50ml of BHI broth which was grown with shaking at 37°C overnight. 40ml of the culture was transferred to an SS34 tube and the cells pelleted at 3000 × g for 10 minutes. Following resuspension of the pellet in 9.5ml of 25% sucrose in TE buffer (10mM Tris, 1mM EDTA (pH8)), 500μl of 10% SDS, 50μl of 20mg/ml proteinase K and 20μl of 10mg/ml RnaseA were added and this mixture incubated in an orbital shaker for 1 hour at 37°C. To this mixture, 1.8ml of 5M NaCl and 1.5ml of a CTAB (N-Cetyl-N,N,N-trimethyl-ammonium bromide) / NaCl solution was added and incubation continued for 20 minutes at 65°C. The DNA was

extracted using phenol/chloroform and precipitated with 0.6 volumes of isopropanol. The resulting DNA was washed in 70% ethanol, dried and resuspended in 2ml of TE buffer.

Screening of genomic library for enzyme activity

The genomic library was cultured on skim milk agar to screen for the presence of a clone displaying protease activity (double strength Columbia agar base (Oxoid Ltd., Basingstoke, Hampshire, U.K.) / 10% skim milk) for 24 hours at 37°C followed by refrigeration at 4°C for one to two days.

A single clone from the genomic library was detected as having activity against skim milk agar. DNA analysis confirmed that the clone contained a fragment of *M. bovis* Dalton 2d genomic DNA approximately 40 kilobases in size. The construct was designated pJF1.

Nucleotide sequence of the *M. bovis* protease clone pJF1

Plasmid and cosmid DNA for automated sequencing was extracted using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) and the Qiagen Plasmid Midi Kit (Qiagen Pty. Ltd., Clifton Hill, Vic, Australia), respectively.

The nucleotide sequence of the insert DNA was determined using the process of "primer walking" (12). This was achieved using synthetic oligonucleotides (Bresatec / Geneworks, Thebarton, SA, Australia) and the dye terminator cycle sequencing ready reaction (Perkin Elmer Corporation, Norwalk, CT, USA). The resulting sequence was analysed on an Applied Biosystems 373A DNA sequencer.

Automated sequencing revealed an open reading frame of 3345bp capable of encoding a protein of 1115 amino acids. The sequence is written in the 5' to 3' direction and is shown in Figure 1 together with the corresponding amino acid sequence which is predicted to encode a protein with a molecular weight of 120kDa. The amino acid sequence is shown in SEQ. ID. NO. 1 and the DNA sequence is shown in SEQ. ID. NO. 2.

The putative start codon for the mature protease protein was identified by the presence of a possible ribosome binding site upstream. This RBS was identified by its similarity to the consensus sequence for the *E. coli* RBS and that previously identified for the *M. bovis* pilin genes (AGGAG) (27)

Due to the secreted nature of the protease, it was assumed that it would contain in its N-terminal sequence a signal peptide which would be used in the secretion of the protein. This analysis was carried out using a prediction program (SignalP) available through the Expasy website (http://www.expasy.ch/tools/), which allows for the identification of prokaryotic signal peptides and predicts possible cleavage sites. This analysis only identified a signal peptide using the start codon indicated in the accompanying protein/DNA sequence.

Sequence comparisons

Comparisons of the deduced amino acid sequence with those in the database were carried out using the BlastX and BlastP programs (13) which are available at http://www.ncbi.nlm.nih.gov.

At the amino acid level, the protease cloned from Dalton 2d displayed the following similarity and identity to the proteins listed.

Organism	Protein	Similarity	Identity
Serratia marcescens	ssp-h2 - serine protease autotransporter	39%	23%
Serratia marcescens	ssp-h1 - serine protease autotransporter	37%	22%
Pseudomonas flourescens	serine protease homologue	34%	20%
Pseudomonas tolaasii	serine protease	35%	21%

More generally the 5' domain of the *M. bovis* protease displays homology to a family of subtilisins (serine proteases) while the 3' region resembles a number of outer membrane proteins.

The *M. bovis* sequence was found to contain a highly proline rich region which distinguished it from all other proteins to which it was closely related.

Protease type encoded by pJF1

In order to identify the type of protease activity encoded by pJF1, a range of specific protease inhibitors were examined for their effect on the expression of the *M. bovis* protease.

The method of Bourgeau *et al.*, (1992) (14) was used to determine inhibitor activity with the following modifications. 100µl of cell free supernatant from a fresh overnight broth culture was mixed with 650µl of 100mM Tris (pH 7.2) and a suitable volume of inhibitor PMSF (phenylmethylsulfonyl fluoride) 5mM; EDTA 5mM; leupeptin 100µg/ml; pepstatin 50µg/ml]. Distilled water was used to make the volume up to 1ml. The mixture was incubated at 37°C for 30 minutes and 10mg of azocoll (Calbiochem, Alexandria, NSW, Australia) was then added. The suspensions were incubated at 37°C for 16 hours and the optical density read at 520nm.

In this way it was confirmed that the activity attributable to the protease encoded by pJF1 was that of a serine protease since PMSF (a serine protease inhibitor) reduced the protease activity of both Dalton 2d and pJF1 to zero.

Conservation of protease in *M. bovis*

Southern hybridisation using an internal fragment of the protease coding region as a probe was carried out to investigate whether the protease was present in strains representing the known *M. bovis* pili serotypes.

Genomic DNA extracted from the representative strains of *M. bovis* (15) was digested with *Xba*I and *Eco*RI and separated using agarose gel electrophoresis. The DNA was transferred to a Hybond N+ filter (Amersham, Little Chalfont, Buckinghamshire, U.K.) using the method described (9). The probe used in the southern hybridisation was a PCR amplified fragment which was internal to the protease coding region. This fragment was labelled with α^{32}P-dATP using the Megaprime labelling system (Amersham, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturers instructions. High stringency conditions were used (hybridisation temperature 68°C; 2 washes at room temperature in 2 × SSC / 0.1% SDS; 1 wash at 68°C in 0.1 × SSC / 0.1% SDS) and the resulting filters were exposed to autoradiographic film (Kodak, Rochester, New York, USA) for 5 to 24 hours before developing.

Results showed that the protease gene cloned in pJF1 is present in all strains of *M. bovis* examined.

EXAMPLE 2

This example describes the cloning and characterisation of a lipase from *Moraxella bovis*.

Bacteria and construction of a plasmid library

Moraxella bovis strain Dalton 2d was a field isolate collected from a bovine eye and characterised by CSIRO Animal Health, Parkville, Australia (6). *Escherichia coli* strain MC1061 has been previously described (16).

All enzymes were purchased from Promega (Madison, WI, USA) except where otherwise noted. General cloning and DNA techniques were as described (9) unless otherwise noted.

A plasmid library was constructed in the cloning vector pBR322 (17). This was done by partially digesting genomic DNA extracted from Dalton 2d (using the CTAB method described in Example 1) with *Sau*3A under conditions that maximised the amount of DNA in the range of 1 to 2kb. This DNA was ligated with pBR322 which had been previously digested with *Bam*HI. The ligated DNA was electroporated (2.5kV, 200Ω and 200μF, for a theoretical time constant of 4.7) into electrocompetent *E. coli* MC1061 cells.

Screening of plasmid library for lipase expression

Following electroporation of the ligated DNA into MC1061 cells, recombinant clones displaying lipase activity were detected by culturing the library for 24 hours at 37°C on media containing Tween 80 [10ml Tween 80 (Sigma, St Louis, MO, USA), 5g NaCl, 3g agar No.1 (Oxoid Ltd., Basingstoke, Hampshire, U.K.), 10g peptone, 0.1g $CaCl_2.H_20$ / litre].

Twenty eight out of 24,000 clones screened were found to be displaying lipase activity. DNA analysis confirmed that all of these clones contained one 5.4kb fragment of DNA in common. One clone was chosen to continue work with and this was designated pMB1.

In some experiments (below), a photometric assay of extracellular lipase activity was performed with p-nitrophenylpalmitate as the substrate (18, 19). Strains of *E. coli* and/or *M. bovis* were grown at 37°C for the required time points. Cell free culture supernatant (100μl) was mixed with 2.4ml of enzyme buffer (19) to assay secreted lipase activity. After 30 minutes incubation at 37°C, the optical density at 410nm was determined.

One enzyme unit was defined as the amount of enzyme that releases 1 nmol of p-nitrophenyl from p-nitrophenylpalmitate ml^{-1} min^{-1}. Under the conditions described by Stuer *et al.*, (18), an optical density at 410nm of 0.041 is equivalent to 1 enzyme unit.

Nucleotide sequence of the *M. bovis* lipase clone pMB1

Plasmid pMB1 was subjected to automated DNA sequencing using the methodology described in Example 1.

This analysis revealed an open reading frame of 1851bp capable of encoding 617 amino acids. The sequence is written in the 5' to 3' direction and is shown in Figure 2 together with the corresponding amino acid sequence that is predicted to encode a protein with a molecular weight of 65.8kDa. The amino acid sequence is shown in SEQ. ID. NO. 3 and the DNA sequence is shown in SEQ. ID. NO. 4.

The techniques set out above in respect of the protease were used to identify the potential start codon for the lipase protein.

Sequence comparisons

Sequence comparisons were made using the methodology described in Example 1.

At the amino acid level, the lipase cloned from *M. bovis* Dalton 2d was shown to display the following similarity and identity to the proteins listed.

Organism	Protein	Similarity	Identity
Xenorhabdus luminescens	triacylglycerol lipase	36%	24%
Pseudomonas putida	hypothetical protein	36%	24%
Salmonella typhimurium	outer membrane esterase	35%	23%
Pseudomonas aeruginosa	lipase / esterase	36%	23%

The *M. bovis* lipase was identified as being a possible new member of the GDSL family (20) of lipolytic enzymes.

N-terminal sequencing carried out on the lipase mature protein

The required strains of *E. coli* were cultured overnight with shaking at 37°C in 500mls of luria broth. The cells were pelleted at 5,000 rpm for 15 mins and the supernatant filtered through a 0.45µm filter. Solid ammonium sulfate was added to the supernatant to 60% saturation (180g / 500ml), and dissolved at 4°C with stirring for 30 minutes. This mixture was left at 4°C overnight and the precipitated proteins pelleted at 7,000 rpm for 30 mins. The proteins were resuspended in 3ml of double distilled water and the solubilised proteins dialysed against double distilled water overnight to remove any salt. The resulting mixture was filtered through a 0.45µm filter and stored at -20°C.

Following separation of the proteins by SDS-PAGE, the proteins were transferred to PVDF membrane and excised. The protein was subjected to automated (Edman degradation) sequence analysis (28) with vapour phase delivery of critical reagents (29) in an automated sequenator (model 470A; Applied Biosystems) (Applied Biosystems Division, Foster City, CA, USA) in conjunction with a PTH amino acid separation system (model 120A PTH analyzer; Applied Biosystems).

Using this technique 17 amino acids with two gaps were identified

K E F S Q V I I F G D S L X D X G (SEQ ID NO:7)

which corresponds exactly with amino acids 26 through to 42 shown on the accompanying sequence. This result also indicated that the protein most likely includes an amino terminal signal peptide which is involved in the secretion of the protein. This amino terminal corresponds to amino acids 1 through to 25 in the accompanying sequence.

Raising antibodies to the lipase in rabbits

Antibody to the recombinant lipase was raised in rabbits by injecting ammonium sulfate precipitated supernatant from *E. coli* MC1061/pMB4. Prior to vaccination, the lipase preparation was inactivated by heating to 90°C for 90min. 30µg of this protein was injected at 2 weekly intervals for 4 weeks. The primary inoculum was emulsified with Freunds complete adjuvant and subsequent vaccinations with Freunds incomplete adjuvant.

Heat stability of *M. bovis* lipase

The recombinant lipase cloned from *M. bovis* Dalton 2d was found to be very heat stable since it required heating at 90°C for 105 minutes for the

activity to be reduced by 97%. Figure 3 illustrates this phenomenon with enzyme activity expressed as "lipase enzyme units" as determined in the extracellular lipase assay.

Relative expression levels of native versus recombinant lipase

An experiment was performed to plot growth rate with lipase production and to compare production of the recombinant lipase from MC1061/pMB1 with that of the native form of the lipase from *M. bovis* Dalton 2d. Figure 4 illustrates that the two strains grow at approximately the same rate but they do not reach the same cell density, with Dalton 2d substantially lower after 9 hours than MC1061/pMB1. Lipase expression levels were greatest from the pMB1 construct in *E. coli* compared to native lipase expression from *M. bovis* Dalton 2d.

This result was further substantiated when proteins from cell-free supernatants of either the *E. coli* clone or *M. bovis* Dalton 2d were ammonium sulfate precipitated and analysed by SDS-PAGE and western blot using antisera to the recombinant heat-deactivated lipase.

Ammonium sulfate precipitated supernatants were prepared from overnight cultures of *E. coli* or *M. bovis* that had been shaken at 37°C in either 500mls of Luria broth or brain heart infusion broth, respectively. Cells were pelleted at 5000 × g for 15 minutes and the supernatant filtered through a 0.45µm filter. Solid ammonium sulfate was added to the supernatant to 60% saturation (180g / 500ml) and dissolved at 4°C with stirring for 30 minutes. This mixture was left at 4°C overnight and the precipitated protein pelleted at 7000 × g for 30 minutes. Proteins were resuspended in 3ml of double distilled water and the solubilised proteins dialysed against double distilled water overnight to remove any salt. The resulting mixture was filtered through a 0.45µm filter and stored at -20°C.

Protein samples (100µl) were prepared for SDS-PAGE by resuspension in 100µl of 2x sample buffer (5ml 0.5M Tris (pH6.8), 8ml 10% SDS, 4ml glycerol, 0.8ml β-mercaptoethanol, 1ml double distilled H_2O, bromophenol blue) and heating to 100°C for 5 minutes. The proteins were separated on a 12.5% polyacrylamide gel using the buffer system of Laemlli (21).

Western blots were carried out according to the method of Towbin *et al.*, (22) and following separation of proteins by SDS-PAGE and transfer to nitrocellulose using the Bio-Rad minicell (Bio-Rad, Hercules, CA, USA)

transfer system. Filters were immunoblotted with the recombinant lipase antiserum (at a concentration of 1/100) which had been adsorbed against MC1061 cells. The antiserum was raised against ammonium sulfate precipitated recombinant lipase which had been heat deactivated (1 hour 45 minutes at 90°C) and used to inoculate rabbits (three doses of 50µg each) at 4 week intervals. Blood samples were collected from the marginal ear vein prior to immunisation and at each vaccination time point.

The results showed a prominent band present in the recombinant lipase positive construct MC1061/pMB1 that is detectable in relatively minor amounts in *M. bovis* Dalton 2d preparation. The protein detected with the antisera was approximately the same size as that of the predicted molecular weight for the *M. bovis* lipase (65.8kDa).

Lipase type encoded by pMB1

Thin layer chromatography (TLC) was used to determine whether the lipase of *M. bovis* Dalton 2d displayed phospholipase activity. Characterisation of phospholipase type essentially followed a previously described method (23) except that the results of separation on Silica Gel 60 plates were visualised by developing with a 10% ethanolic solution of molybdophosphoric acid at 100°C. All reagents used were purchased from Sigma (Sigma, St Louis, MO, USA).

TLC determined that the *M. bovis* lipase displayed the same enzyme specificity as that of a commercially-available phospholipase B when lysophosphatidylcholine and phosphatidylcholine were used as substrates (data not shown).

Conservation of lipase among *M. bovis*

A southern blot using an internal fragment of the Dalton 2d lipase coding region was used to investigate whether the lipase gene was present in strains of *M. bovis* representing the known pilus serotypes.

Genomic DNA extracted from the strains of *M. bovis* representing each of the known pilus serotypes (15) was digested with *Hin*dIII and separated using agarose gel electrophoresis. The DNA was transferred to a Hybond N+ filter (Amersham, Little Chalfont, Buckinghamshire, U.K.) using a previously described method (9). The probe used in the southern hybridisation was a *Hin*dIII fragment that contained sequence internal to the lipase coding

region. This fragment was labelled with α^{32}P-dATP using the Megaprime labelling system (Amersham, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturers instructions. High stringency conditions were used (hybridisation temperature 68°C; 2 washes at room temperature in 2 × SSC / 0.1% SDS; 1 wash at 68°C in 0.1 × SSC / 0.1% SDS) and the resulting filters were exposed to autoradiographic film (Kodak, Rochester, New York, USA) for 5 to 24 hours before developing.

Results showed that the lipase gene is present in all strains of *M. bovis* examined.

To confirm whether or not the lipase gene was expressed in each of the serotype representative strains, antisera raised against recombinant heat deactivated lipase was used in a western blot analysis of whole cell preparations. Results showed that the lipase was indeed being expressed by all of these *M. bovis* strains.

EXAMPLE 3

Bacteria and construction of a haemolysin clone

Moraxella bovis strain Dalton 2d was a field isolate collected from a bovine eye and characterised by CSIRO Animal Health, Parkville, Australia (6).

All of the *M. bovis* strains representative of the known pilus serotypes express a haemolytic activity that is detected on horse blood agar.

Escherichia coli strain degP4::Tn5 has a leaky outer membrane and is defective in proteolysis and has been previously described (24).
All enzymes were purchased from Promega (Madison, WI, USA) except where otherwise noted.

General cloning and DNA techniques were as described (9) unless otherwise noted.

A *phoA* fusion technique that allows for the identification of exported proteins (25) was utilised with some modifications. Genomic DNA from *M. bovis* Dalton 2d (prepared using the CTAB method described in Example 1) was partially digested with *Sau*3A. Restricted DNA was ligated with a series of vectors that allow fusions with an alkaline phosphatase gene in three different reading frames. The ligated DNA was electroporated into *E. coli* degP4::Tn5 and the resulting clones screened on Luria agar containing

ampicillin (50μg/ml) and X-P (200μg/ml) (5-bromo-3-chloro-indolyl phosphate). Selection of clones relies on the observation that if the fragment is cloned in frame and contains an export sequence the resulting colony will be blue in colour. The leaky *E. coli* strain allows the outer membrane-bound proteins and secreted proteins (both fused with *phoA*) to be distinguished from non-secreted fusion proteins.

Sequencing of the *M. bovis* haemolysin determinant

Clones selected for the presence of a secreted or outer membrane protein gene sequence were subjected to automated DNA sequencing using the methods described in Example 1. One of these clones, pMbh1, was found to contain 200bp of DNA which displayed high homology to the A subunit of other RTX toxins. Inverse PCR and degenerate oligonucleotides were utilised to obtain the sequence of the entire A subunit. The open reading frame of 2784bp was capable of encoding 928 amino acids. The sequence is written in the 5' to 3' direction and is shown in Figure 5 together with the corresponding amino acid sequence that is predicted to encode a protein with a molecular weight of 98.8kDa. The amino acid sequence is shown in SEQ. ID. NO. 5 and the DNA sequence is shown in SEQ. ID. NO. 6.

The putative start codon was identified using the RBS technique outlined above. A signal peptide analysis was not carried out as the A subunit is not secreted on its own. However as the protein sequence of these proteins (RTX) is quite highly conserved, on amino acid homologies alone this start codon was the one of choice.

Sequence homology

At the amino acid level the *M. bovis* Dalton 2d haemolysin gene product shows striking similarity to the A subunit of the of several RTX and other haemolysins as shown in the following table.

Organism	Protein	Similarity	Identity
Pasteurella haemolytica	LktA protein (leukotoxin)	68%	50%
Actinobacillus pleuropneumoniae	RTX toxin determinant	68%	48%
Eschericlua coli	Haemolysin - plasmid	58%	43%
E. coli	Haemolysin - chromosomal	58%	43%

Functional complementation by the *M. bovis* haemolysin

A construct which expressed the chromosomal-borne haemolysin of *E. coli* was obtained (pLG900; generated by combining the two plasmids pLG575 (26) and pLG816 (*hlyC* and *hlyA* cloned into pBluescriptSK). pLG900 comprises the four genes of the RTX operon, *hlyC, hlyA, hlyB, hlyD,* cloned into pBluescriptSK and is capable of conferring a haemolytic phenotype on *E. coli* cells that were previously non-haemolytic. The A subunit (*hlyA*) of this construct was mutated such that it was no longer able to be expressed but the other genes involved in the operon (*hlyB, hlyC* and *hlyD*) remained intact. The *E. coli* strain containing this construct (pLG900 / *hlyA* negative) was no longer haemolytic. However, the haemolytic phenotype was restored by providing *in trans* the cloned haemolysin subunit gene from *M. bovis* Dalton 2d. Thus it was confirmed that the cloned *M. bovis* haemolysin gene encoded a structural subunit that was most probably a member of the RTX family of haemolytic enzymes.

Further sequence analysis has established that, like other members of the family, the *M. bovis* RTX A subunit gene is flanked by DNA sequences capable of encoding the RTX B,C and D proteins.

Conservation of the RTX A subunit among *M. bovis*

To determine whether the gene for the RTX A subunit was present in *M. bovis* strains representing the known pilus serotypes, a southern hybridisation analysis was performed using the coding region of the RTX A subunit as a probe.

Genomic DNA extracted from the seven serotype strains of *M. bovis* (15) was digested with *Eco*RV and separated using agarose gel electrophoresis. The DNA was transferred to a Hybond N+ filter (Amersham,

Little Chalfont, Buckinghamshire, U.K.) using a previously described method (9). The probe used was a PCR amplified product that contained all of the coding region from the A subunit of the RTX haemolysin of *M. bovis*. This fragment was labelled with α^{32}P-dATP using the Megaprime labelling system (Amersham, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturers instructions. High stringency conditions were used (hybridisation temperature 68°C; 2 washes at room temperature in 2 × SSC / 0.1% SDS; 1 wash at 68°C in 0.1 × SSC / 0.1% SDS) and the resulting filters were exposed to autoradiographic film (Kodak, Rochester, New York, USA) for 5 to 24 hours before developing.

Results showed that the gene encoding the RTX A haemolysin subunit was conserved in all seven representative strains of *M. bovis* examined. Interestingly, each of these strains is known to display the haemolytic phenotype on horse blood agar. In contrast, the non-haemolytic *M. bovis* strain Gordon 26L3 did not hybridise to the RTX A gene probe possibly suggesting that *M. bovis* contains only a single structural gene responsible for the haemolytic phenotype detected on horse blood agar.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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